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Watermelon glyoxysomal malate dehydrogenase is sorted to peroxisomes of the methylotrophic yeast, *Hansenula polymorpha*

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We have studied the fate of the watermelon (*Citrullus vulgaris* Schrad.) glyoxysomal enzyme, malate dehydrogenase (gMDH), after synthesis in the methylotrophic yeast, *Hansenula polymorpha*. The gene encoding the precursor form of gMDH (pre-gMDH) was cloned in an *H. polymorpha* expression vector downstream of the inducible *H. polymorpha* alcohol oxidase promoter. During methylotrophic growth, pre-gMDH was synthesized and imported into peroxisomes, where it was enzymatically active. The apparent molecular mass of the protein located in *H. polymorpha* peroxisomes was equal to that of pre-gMDH (41 kDa), indicating that N-terminal processing of the transit peptide had not occurred in the yeast.

Malate dehydrogenase; Glyoxysome; Peroxisome; *Hansenula polymorpha*; *Citrullus vulgaris*; Protein targeting

1. INTRODUCTION

In the methylotrophic yeast, *Hansenula polymorpha*, the proliferation and metabolic function of peroxisomes can readily be prescribed by manipulating growth conditions [1]. This property renders *H. polymorpha* an attractive model organism for the molecular analysis of peroxisome biogenesis and function [2,3]. So far, two peroxisomal-targeting signals (PTS) have been identified, which direct precursors of peroxisomal matrix proteins to the correct target. The first one (PTS1) is the conserved C-terminal tripeptide, SKL, which was identified in firefly luciferase and is shown to be highly conserved among peroxisomal matrix proteins from various eukaryotes, like mammals, plants and yeasts, including *H. polymorpha* [4]. In *H. polymorpha*, three peroxisomal enzymes (alcohol oxidase, dihydroxy acetone synthase and catalase) contain a PTS1-like motif (-ARF, -NKL and -SKI, respectively), which have been shown to function as the targeting signal [5,6]. PTS2 has been identified in the N-terminal presequence of rat peroxisomal 3-ketoacyl-CoA-thiolases. These presequences are cleaved-off upon translocation of the proteins into the peroxisomal matrix [7]. A comparable signal is assumed to function for watermelon glyoxysomal malate dehydrogenase (gMDH) [8]. Evidence is accumulating now that at least two distinct import pathways may exist for peroxisomal proteins, which are specific for proteins containing either PTS1 or PTS2 [2,9].

In *H. polymorpha*, none of the so far identified peroxi-

somal proteins is synthesized as a precursor containing an N-terminal, cleavable presequence similar to those of rat thiolases and watermelon gMDH. This prompted us to study whether the molecular mechanisms involved in import/processing of such proteins are more universally conserved and functioning in *H. polymorpha*. For this purpose we introduced the gene encoding the precursor of watermelon glyoxysomal malate dehydrogenase (pre-gMDH) into *H. polymorpha* and studied the fate of the synthesized pre-gMDH protein in this organism. The results of these studies are presented in this paper.

2. MATERIALS AND METHODS

2.1. Yeast strains and growth conditions

Untransformed *H. polymorpha* A16 [10] and transformants carrying the gene encoding watermelon malate dehydrogenase under the control of the *H. polymorpha* alcohol oxidase promoter (P_{MOX}; see below) were grown in batch cultures in mineral medium [11] containing methanol (0.5%) or a mixture of glycerol (0.1%) and methanol (0.5%) as carbon sources.

2.2. Plasmid constructions

Escherichia coli DH1 [*sup* E44 *hsd* R17 *rec* A1 *end* A1 *gyr* A96t *thi* -1 *rel* A1] was used for plasmid amplification and grown in LB medium. All recombinant DNA manipulations were performed using standard methods [12]. A 1.3 kb *Not*I (sticky ends filled in)-*Sal*I DNA fragment from pGEMEX-MDH [8], containing the entire cDNA coding region for the precursor of watermelon glyoxysomal malate dehydrogenase (pre-gMDH), was inserted into the *H. polymorpha* expression vector, pHIPX2 (K.N. Faber, unpublished). The vector was digested with *Hind*III (sticky ends filled in) and partially with *Sal*I. Those fragments, digested at the *Sal*I site 19 nucleotides 5' from the *Hind*III site, were selected and used for the insertion of the pre-gMDH gene. The resulting plasmid carrying the pre-gMDH gene under the control of the *H. polymorpha* P_{MOX} was designated pGF159 (Fig. 1).

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2.3. Yeast transformation and transformant selection

H. polymorpha A16 was transformed with plasmid pGF159 using a recently developed electrotransformation procedure (Faber et al., submitted). Prior to transformation the expression vector was linearized in P_{mox} by *Stu*I digestion, forcing integration of the plasmid DNA at the homologous locus on the genome [13]. Integrants were selected by growth of transformants for at least 40 generations on non-selective medium and further checked by Southern blot analysis (not shown). Strains M1 (2–3 copies of PGF159) and M3 (one copy) were used for further studies.

2.4. Biochemical methods

Crude extracts were prepared as described previously [14]. Protein concentrations [15] and activities of alcohol oxidase [14], catalase [16] and malate dehydrogenase [17] were assayed as described. Enzyme activities are expressed as μmol substrate consumed or product formed $\cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$, except for catalase which is expressed as $\Delta A_{240} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$. Cells were fractionated by differential and sucrose density centrifugation of homogenized protoplasts [14]. Peroxisomal and mitochondrial peak fractions were identified by measuring organellar marker enzymes [14]. The crude extracts and subcellular fractions were analyzed by SDS-PAGE [18], followed by Western blotting, using the protoblot immunoblotting system (Promega Biotech) and polyclonal antibodies generated against watermelon glyoxysomal MDH (α -gMDH) and watermelon mitochondrial MDH (α -mMDH). α -gMDH does not recognize any proteins in the crude extracts of untransformed *H. polymorpha* A16. The α -mMDH antibody cross-reacts with the *H. polymorpha* mitochondrial MDH and with a cytosolic protein; in addition they show weak cross-reactivity with watermelon gMDH, expressed in *H. polymorpha* transformants.

2.5. Electron microscopy

Intact cells and spheroplasts were prepared for electron microscopy as described before [19]. Immunocytochemistry was performed on ultrathin sections of Lowicryl K_4 M-embedded cells by the protein A/gold method [20], using polyclonal antibodies raised against watermelon glyoxysomal MDH (α -gMDH).

3. RESULTS AND DISCUSSION

3.1. Growth and gMDH expression

Cells of both transformed strains, M1 and M3, grew

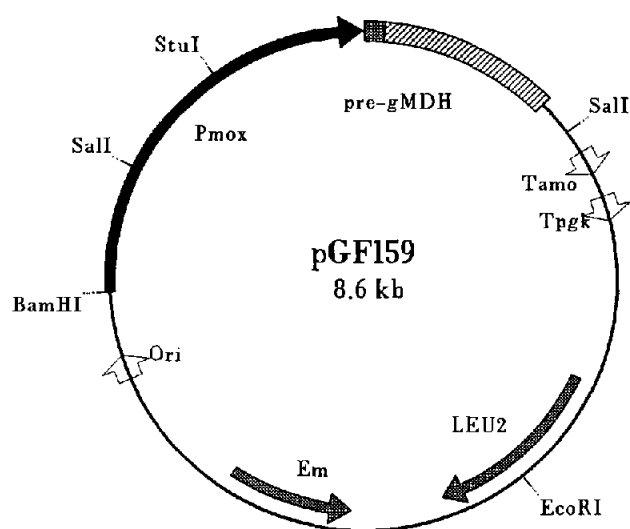


Fig. 1. Physical map of the vector, pGF159, containing the entire gene encoding the precursor of watermelon glyoxysomal MDH (pre-gMDH) under the control of the alcohol oxidase promoter (P_{mox}).

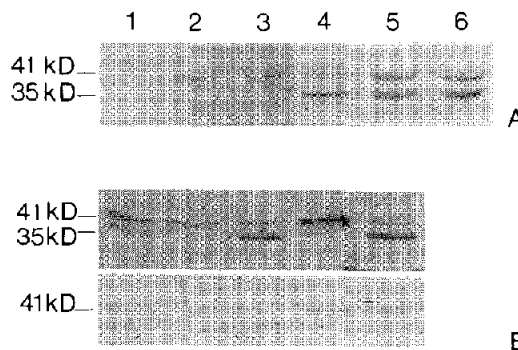


Fig. 2. Western blots of crude extracts and subcellular fractions of wild-type control and transformed cells of *H. polymorpha*, synthesizing watermelon gMDH. (A) Western blots prepared from crude extracts of methanol-grown control cells (strain A16; lanes 1 and 4) and transformants M1 (multicopy; lanes 2 and 5) and M3 (single copy; lanes 3 and 6). 30 μg of protein was loaded in each lane. Antibodies raised against either watermelon glyoxysomal MDH (α -gMDH; lanes 1–3) or mitochondrial MDH (α -mMDH; lanes 4–6) were used. (B) Cells of the transformed strain, M1, were fractionated by differential centrifugation of homogenized protoplasts into a cytosolic fraction (30,000 $\times g$ supernatant; lane 2) and an organellar pellet (30,000 $\times g$ pellet; lane 3). The organellar fraction was further separated by sucrose gradient centrifugation into purified peroxisomal (lanes 4) and mitochondrial (lanes 5) fractions. Lane 1: crude extracts of whole cells. Each lane was loaded with 30 μg of protein for SDS-PAGE, followed by Western blotting. (Upper panel, α -mMDH antibodies; lower panel, α -gMDH antibodies.)

well on various carbon sources, including methanol, at rates comparable to the parental strain, A16. For the synthesis of gMDH, cells of the above strains were grown on methanol in order to create conditions in which the alcohol oxidase promoter (P_{mox}) is fully depressed [11]. In crude extracts, prepared from the transformed strains, M1 (2–3 copies) and M3 (single copy), the total MDH activity was increased (specific activity of 31 and 27 $\text{U} \cdot \text{mg protein}^{-1}$, respectively), compared to the untransformed host strain, A16 (23 $\text{U} \cdot \text{mg protein}^{-1}$), suggesting that the watermelon gMDH is synthesized and enzymatically active in both transformants. Further evidence for the synthesis of pre-gMDH in the heterologous host, *H. polymorpha*, was obtained by Western blot analysis (Fig. 2A). For this purpose two polyclonal antisera were available, raised against watermelon gMDH (α -gMDH) and the mitochondrial isoenzyme (α -mMDH). In watermelon these antisera recognize the corresponding MDH but show weak cross-reactivity with the other MDH isoenzymes (data not shown).

In crude extracts of the untransformed parental strain, A16, the α -gMDH antibodies did not recognize any protein, and thus also do not recognize homologous *H. polymorpha* MDH's (Fig. 2A). However, using these antibodies, a single protein band (apparent molecular weight of 41 kDa) was detected in crude extracts prepared from methanol-grown cells of the transformed strains, M1 and M3 (Fig. 2A). The intensity of the 41

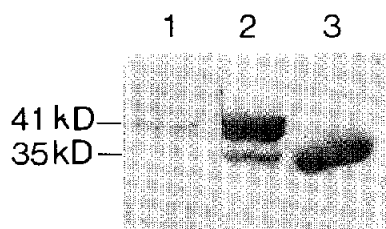


Fig. 3. Western blot of the peroxisomal peak fraction of transformant M1 (lane 1) and extracts prepared from *E. coli* cells which over-expressed either pre-gMDH (lane 2) or mature gMDH (lane 3). The blot was labeled with α -gMDH antibodies and clearly shows that the protein synthesized in *H. polymorpha* (lane 1) has the same apparent molecular weight as pre-gMDH (lane 2), and therefore is not processed into the mature watermelon glyoxysomal MDH (lane 3).

kDa band was enhanced in extracts from M1, as expected from the observed specific activities (see above), probably reflecting an enhanced rate of gMDH synthesis due to the higher number of expression vectors integrated in the genome of strain M1.

Two major bands (41 and 35 kDa, respectively) were observed on Western blots when the α -mMDH antibodies were used in the above experiments. The lower band most probably represents the *H. polymorpha* mitochondrial MDH, whereas in the upper band, the heterologous gMDH and an endogenous protein, most probably cytosolic MDH, overlap. The increased intensity of the upper 41 kDa band in extracts from strains M1 and M3, compared to those from strain A16, again indicates expression of the watermelon gMDH gene in both transformants.

3.2. Pre-gMDH is located in peroxisomes and enzymatically active

For the biochemical localization of gMDH, cell fractionation studies were performed using cells from strain M1 and the parental strain A16, grown on a mixture of glycerol/methanol. After sucrose density gradient centrifugation of the $30,000 \times g$ pellets, obtained after differential centrifugation of homogenized protoplasts, highly purified fractions of mitochondria (located at 45% sucrose) and peroxisomes (located at 53% sucrose) were obtained. The assignment of the organelles was based on the distribution of the activities of their respective marker enzymes, cytochrome *c* oxidase and catalase (data not shown). Their purity was confirmed by electron microscopy (data not shown). Western blot analysis of the various fractions using α -gMDH antibodies, which only recognize the heterologous gMDH protein in *H. polymorpha*, revealed that watermelon gMDH co-sedimented with catalase, indicating its peroxisomal location (Fig. 2B). The presence of minor bands in both the mitochondrial peak as well as in the $30,000 \times g$ supernatant fraction (S_4) might be explained by partial leakage of the gMDH protein from intact peroxisomes during the isolation procedure, a phenomenon which has been described before for peroxisomal catalase [19].

As expected, the 35 kDa protein cross-reacting with α -mMDH antibodies co-fractionated with purified mitochondria. This band was completely absent in the peroxisomal peak fractions, confirming the high purity of these fractions (Fig. 2B).

Biochemically, the specific activity of MDH in the peroxisomal peak fraction of strain M1 is 9-fold increased compared to the control strain, A16 (Table I). The latter activity most probably represents the homologous MDH activity in purified peroxisomal fractions of glycerol/methanol-grown cells of strain A16. Based on the electrophoretic mobility of the protein recognized by α -gMDH antibodies in crude extracts and purified peroxisomal fractions of strain M1, the calculated apparent molecular weight is 41 kDa (Fig. 2A,B). The reported values for pre-gMDH after in vitro transcription/translation range from 37 to 41 kDa; the apparent molecular weight of the mature, processed form is approximately 33 kDa [8,21]. This indicates that in *H. polymorpha*, cleavage of the 37 residue-long N-terminal presequence has not occurred. The latter was confirmed by comparing the electrophoretic mobility of gMDH in peroxisomal peak fractions, isolated from cells of strain M1, with crude extracts prepared from *E. coli*, over-expressing either precursor or mature gMDH. As is evident from Fig. 3, watermelon gMDH synthesized in *H. polymorpha* indeed co migrates with the gMDH precursor.

3.3. Ultrastructural analysis

The subcellular morphology of cells of the parental strain, A16, and the transformant, M1, grown in methanol-containing medium, was studied by electron microscopy. Analysis of ultrathin sections of KMnO_4 -fixed cells of strain M1 indicated that both the average number and size of the peroxisomes in these cells were virtually unaltered compared to cells of A16 (Fig. 4A). The substructure of the organelles, examined in thin sections of glutaraldehyde/osmium-fixed spheroplasts (Fig. 4B), was also highly comparable in that they contained large alcohol oxidase crystalloids [11]. However, in peroxisomes present in cells from strain M1, electron-dense material was observed in the small region between the crystalloid and the surrounding membranes (Fig. 5A, arrow), which is absent in the A16 control and may represent imported gMDH protein. The latter is con-

Table I

Specific activities of alcohol oxidase (AO), catalase and MDH in peroxisomal peak fractions obtained after differential and sucrose gradient centrifugation of homogenized protoplasts

Strain	AO	Catalase	MDH
A16	2.3	45.5	4.8
M1	2.6	51.9	43.7

A16, parental strain; M1, gMDH transformant of *H. polymorpha*.

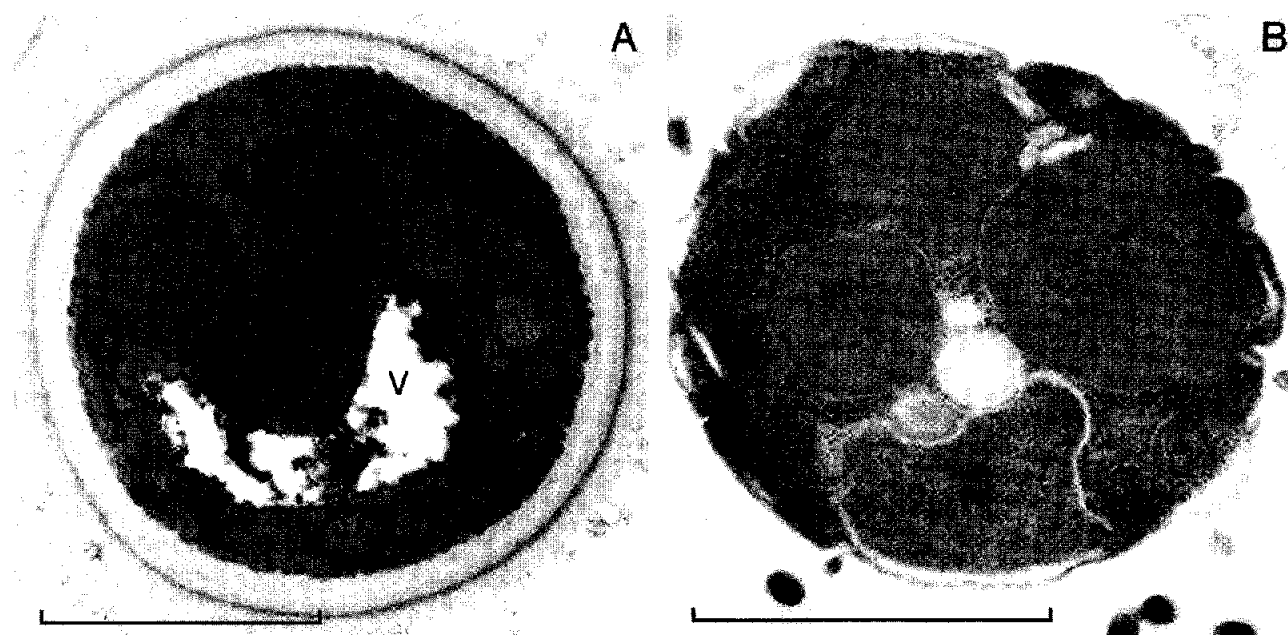


Fig. 4. Ultrathin sections of methanol-grown cells of the transformed *H. polymorpha* (strain M1) to show the overall subcellular morphology (A; KMnO_4) and crystalline substructure of peroxisomes (B; protoplast, glutaraldehyde/ OsO_4). P, peroxisome; M, mitochondrion; V, vacuole; N, nucleus. Bar = 1.0 μm unless otherwise stated.

firmed by the results of the immunolabelling experiments (Fig. 5B,C). Using the α -gMDH antibodies, which only recognize the watermelon MDH protein, specific labeling was confined to the peroxisomal matrix, predominantly located at the periphery of the organelles. An identical localization has been observed for peroxisomal catalase in fully crystalline peroxisomes, present in methanol-limited *H. polymorpha* wild-type cells. Apparently, like catalase [19], gMDH is not able to diffuse into the intracrystalline spaces of the alcohol oxidase crystalloid, as observed for several other peroxisomal matrix enzymes [22]. However, the above lo-

cation of gMDH in peroxisomes of *H. polymorpha* could explain the observed preferential leakage of gMDH protein during organellar purification, similar to that described for peroxisomal catalase [19].

3.4. Concluding remarks

Summarizing, the above results indicate that watermelon pre-gMDH is correctly, targeted to *H. polymorpha* peroxisomes and translocated across the peroxisomal membrane. This implies that the targeting signal of the plant glycoxysomal enzyme also functions in *H. polymorpha*. In this respect it is relevant to mention

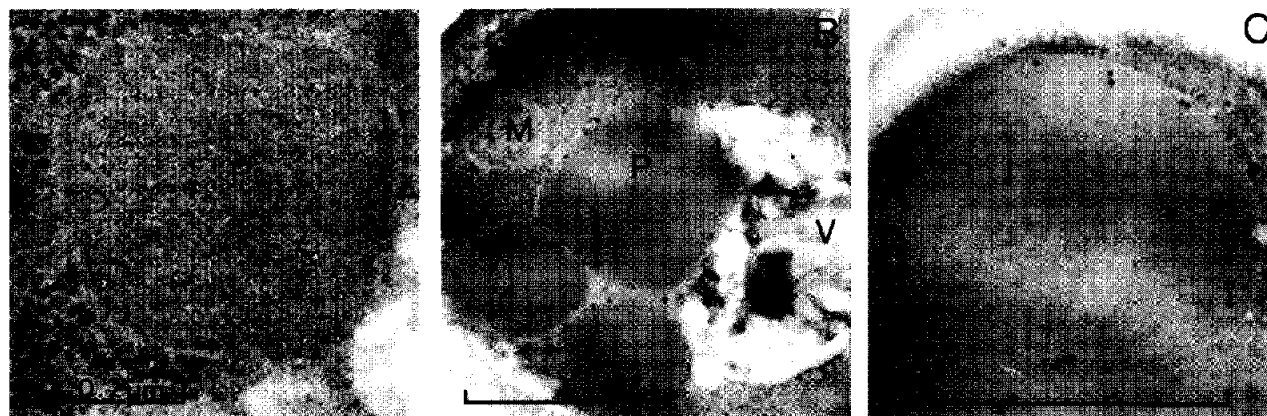


Fig. 5. (A) High magnification of the peroxisome, indicated by 'P' in Fig. 4B, to show the presence of electron-dense material in the narrow zone between the alcohol oxidase crystalloid and the peroxisomal membrane (arrows). Immunocytochemical experiments using α -gMDH antibodies and protein A/gold revealed that the specific labeling was confined to peroxisomes and predominantly located at the periphery (B). As is evident from the high magnification shown in C, labeling is indeed located in the organellar matrix, indicating that gMDH is properly imported. P, peroxisome; M, mitochondrion; V, vacuole; N, nucleus. Bar = 1.0 μm unless otherwise stated.

that the N-terminal part of the presequences of rat thiolases A and B, which has been shown to contain PTS2, and watermelon gMDH, contain significant positional identities. Surprisingly, a considerable identity was also found with the first 11 N-terminal amino acids of the *H. polymorpha* peroxisomal protein amine oxidase [23]. Together with our earlier finding that the C-terminus of amine oxidase does not contain targeting information, it is therefore likely that a PTS2-like signal is responsible for amine oxidase targeting. Moreover, mutation analyses revealed that the targeting information of amine oxidase does not reside at the C-terminus [24].

Interestingly, the peroxisomal MDH of the yeast, *Saccharomyces cerevisiae*, does contain the consensus C-terminal PTS1 (SKL) [25]. Thus, one and the same peroxisomal enzyme may apparently contain different peroxisomal targeting signals, depending on the organism examined.

In *H. polymorpha*, processing of the N-terminal presequence of gMDH was not observed, suggesting that a specific peroxisomal peptidase, required for this processing event, is absent or not functional in the case of heterologous gMDH. So far, proteolytic processing of peroxisomal proteins has not been observed in yeast, but only encountered in higher eukaryotes like mammals and plants. Despite the fact that processing of pre-gMDH did not occur, the pre-gMDH protein displayed enzyme activity in *H. polymorpha*. Also, pre-gMDH protein expressed in *E. coli* is enzymatically active (C. Gelt, unpublished results). In watermelon glyoxysomes the active enzyme is an oligomeric protein, consisting of two identical subunits of the mature size. Whether pre-MDH is active in *H. polymorpha* peroxisomes as a monomer or dimer is still unclear.

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